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Short sequence- and promoter paper

Cloning and promoter analysis of the cotton lipid transfer protein gene *Ltp3*[1]

Hsi-Chou Liu [a], Roy G. Creech [b], Johnie N. Jenkins [b,c], Din-Pow Ma [a,*]

[a] *Department of Biochemistry and Molecular Biology, Box 9650, Mississippi State University, Mississippi State, MS 39762, USA*
[b] *Department of Plant and Soil Sciences, Mississippi State University, Mississippi State, MS 39762, USA*
[c] *Crop Science Research Laboratory, USDA/ARS, Mississippi State, MS 39762, USA*

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Abstract

A cotton *Ltp3* gene and its 5′ and 3′ flanking regions have been cloned with a PCR-based genomic DNA walking method. The amplified 2.6 kb DNA fragment contains sequences corresponding to GH3 cDNA which has been shown to encode a lipid transfer protein (LTP3). The gene has an intron of 80 bp which is located in the region corresponding to the C-terminus of LTP3. The *Ltp3* promoter was systematically analyzed in transgenic tobacco plants by employing the *Escherichia coli* β-glucuronidase gene (GUS) as a reporter. The results of histochemical and fluorogenic GUS assays indicate that the 5′ flanking region of the *Ltp3* gene contains *cis*-elements conferring the trichome specific activity of *Ltp3* promoter.

Keywords: Genomic DNA walking; Intron; Polymerase chain reaction; Reporter gene

A cotton fiber cDNA (GH3) encoding a lipid transfer protein (LTP3) was previously isolated in our laboratory using a differential screening method [1]. Northern analysis data indicate that the gene corresponding to GH3, *Ltp3*, is specifically expressed in fiber cells and is developmentally regulated. The *Ltp3* RNA transcript level reaches a maximum at 15 days post-anthesis (DPA) at the late fiber elongation stage. From the *Ltp3* expression pattern and the general functions of plant LTPs, we proposed that LTP3 is involved in cutin synthesis during the fiber primary cell wall synthesis stage. Due to its fiber specific nature, the *Ltp3* gene promoter will be useful in cotton genetic engineering to introduce novel traits into fiber. In this study, the *Ltp3* gene along with its 5′ and 3′ flanking regions was cloned by using a PCR-based genomic DNA walking method [2]. The *Ltp3* 5′ flanking region containing the promoter activity was subsequently analyzed with the *Escherichia coli* GUS gene as a reporter.

Cotton (*Gossypium hirsutum* L. DES119) genomic DNA was isolated by a method according to Paterson et al. [3]. The genomic DNA was digested with *Xba*I, filled in with T4 DNA polymerase, and then ligated with the Marathon cDNA adapter (Clontech) which consists of two oligonucleotides (5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′ and 5′-PO_4-ACCTGCCC-NH_2-3′). The adapter-ligated genomic DNA was

* Corresponding author. Fax: +1-662-3258664; E-mail: dm1@ra.msstate.edu

[1] The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Databases under the accession number AF228333.

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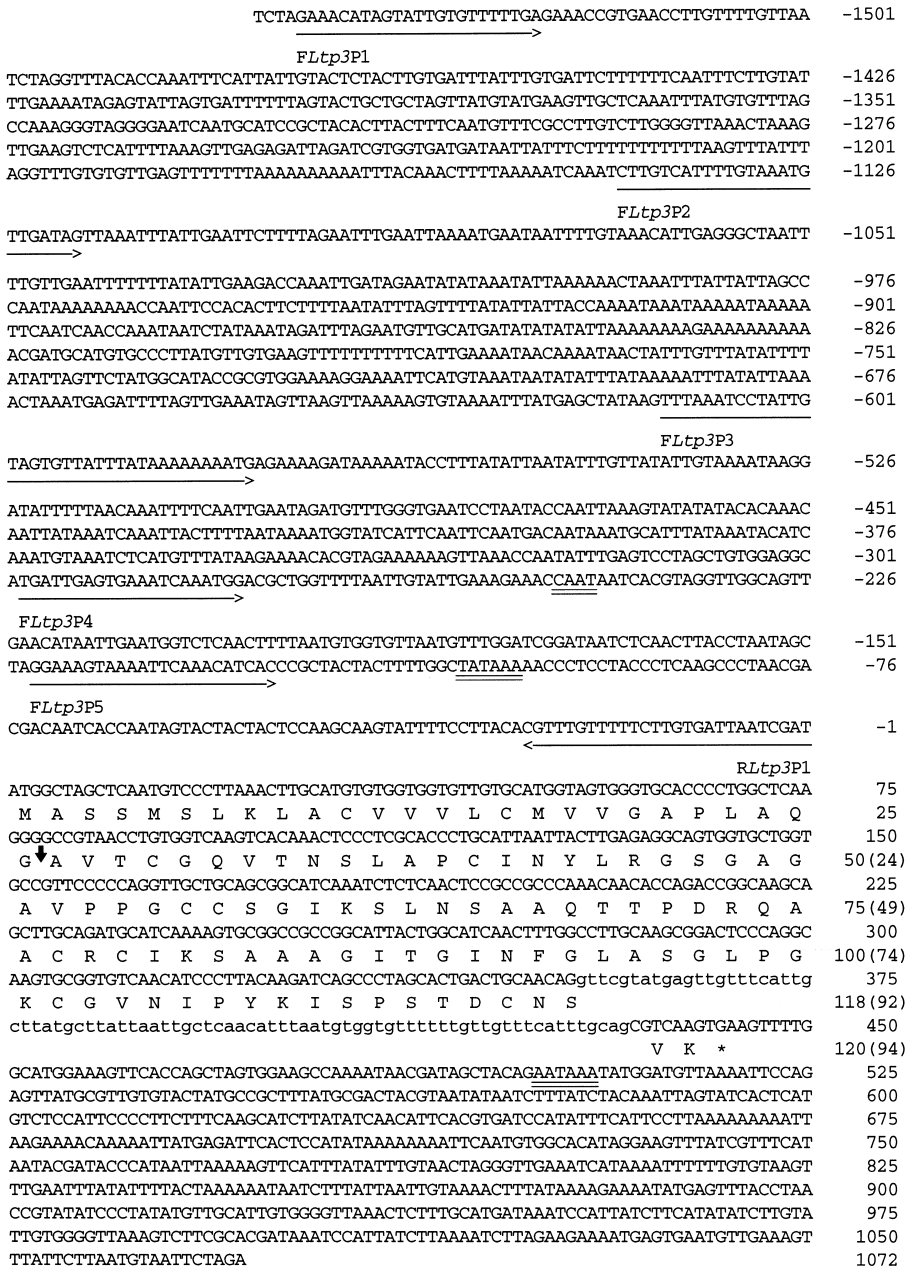

Fig. 1. Nucleotide and derived amino acid sequences of the cotton *Ltp3* gene. The basal promoter elements (TATA and CAAT boxes) and the polyadenylation signal (AATAAA) are double-underlined. The sequence of the 80 bp intron is presented in lowercase letters. The translational stop codon is marked with an asterisk. The numbers in parentheses denote the aa positions in mature LTP3 protein. The A nucleotide of the translational initiation codon ATG is assigned as position 1 in the nucleotide sequence, and the nucleotide positions upstream of position 1 are presented with minus numbers. The sequence and direction of six primers used in PCR amplification to generate the five *Ltp3* promoters with different lengths (see Fig. 2) are represented by long arrows. The cleavage site of the N-terminal signal peptide is marked by a short arrow.

used as a template in PCR amplification [4] of the 5′-flanking region of the *Ltp3* gene by *TaqPlus* DNA polymerase (Stratagene) using the GH3-specific primer (5′-CGCTCTAGATACTAATTTGTAGATAAAG-3′) and adapter primer 1 (AP1: 5′-CCATCCTAATACGACTCACTATAGGGC-3′). The PCR product was diluted 50 fold and then used as the template for the second (nested) PCR amplifica-

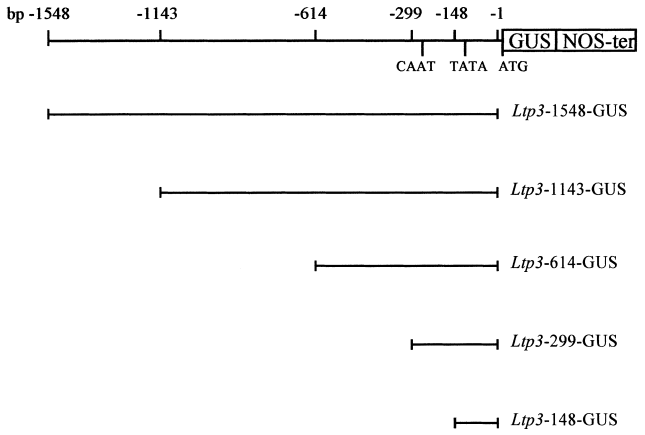

Fig. 2. Schematic diagram of the *Ltp3* promoter-GUS constructs. Five *Ltp3* promoters (*Ltp3*-1548, *Ltp3*-1143, *Ltp3*-614, *Ltp3*-299, and *Ltp3*-148) with different lengths were amplified by PCRs and fused to the GUS gene in plasmid pBI101. Noster represents the transcriptional terminator of the nopaline synthase gene. The initiation codon of GUS is 27 bp downstream from the −1 position.

tion using GH3-specific primer (5′-TCGTCTAGAGCGGCATAGTACACAACGCAT-3′) and adapter primer 2 (AP2: 5′-ACTCACTATAGGGCTCGAGCGGC-3′). The *Ltp3* 3′-flanking region was amplified by the primary and nested PCRs using AP1/AP2 and GH3-specific primers (5′-ATGGATCCATGGCTAGCTCAATGTCCC-3′ for primary PCR and 5′-AAGGATCCGCCGTAACCTGTGGTCAAG-3′ for nested PCR). The PCR-amplified DNA fragments were cloned into M13mp18/19 vectors [5] and sequenced using the PRISM Ready Reaction DyeDeoxy Termination cycle sequencing kit with the ABI PRISM 310 Genetic Analyzer (Perkin Elmer). Two primers were then synthesized based on the sequences of the 5′ and 3′ flanking regions of the *Ltp3* gene, and used in PCR amplification of the *Ltp3* genomic DNA sequence using *Pfu* DNA polymerase (Stratagene). The nucleotide sequence of the 2.6 kb *Xba*I DNA fragment containing the *Ltp3* gene is shown in Fig. 1. The 5′ flanking region of the *Ltp3* gene contains CAAT and TATA boxes which serve as basal promoter elements for transcription. A polyadenylation signal (AATAAA) is located in the 3′-flanking region. Similar to the *Ltp6* gene [6], the *Ltp3* gene has a single intron located in the region corresponding to the C-terminus of the encoded protein. The *Ltp3* intron also contains A+T rich sequences and has the conserved GT and AG sequences at the 5′ and 3′ splicing sites. However, the intron size (80 bp) of *Ltp3* is smaller than that (136 bp) of *Ltp6*. Six nucleotides in the determined *Ltp3* genomic sequence were found to be different from the corresponding ones at nucleotide positions 20, 49, 166, 250, 251, and 292 of the GH3 cDNA. Since *Pfu* DNA polymerase possesses the 3′ to 5′ proofreading exonuclease activity, these sequence differences might be due to amplification errors introduced into GH3 cDNA by *Taq* DNA polymerase, which does not possess the proofreading activity. From the genomic sequence, the aa residues of LTP3 at positions 4, 32, and 46 in the mature protein should be Cys, Ser, and Asp, respectively, instead of Ser, Thr, and Val as deduced from the GH3 cDNA sequence [1]. These corrections indicate that mature LTP3 protein has eight conserved Cys residues (Cys^4, Cys^{14}, Cys^{30}, Cys^{31}, Cys^{51}, Cys^{53}, Cys^{76}, and Cys^{90}) for the formation of four disulfide bonds (Cys^4–Cys^{53}, Cys^{14}–Cys^{30}, Cys^{31}–Cys^{76}, and Cys^{51}–Cys^{90}) as revealed in the structural study of a mature maize LTP by X-ray crystallography [7], rather than seven Cys residues as previously deduced from the GH3 cDNA sequence.

The 5′ flanking region of the *Ltp3* gene was PCR amplified with six primers (F*Ltp3*P1, F*Ltp3*P2, F*Ltp3*P3, F*Ltp3*P4, F*Ltp3*P5, and R*Ltp3*P1; Fig. 1) to generate five *Ltp3* promoters (*Ltp3*-1548, *Ltp3*-1143, *Ltp3*-614, *Ltp3*-299, and *Ltp3*-148) with different lengths (Fig. 2). Each PCR-amplified *Ltp3* promoter was cloned as a *Hin*dIII–*Xba*I fragment into the binary plasmid pBI101 (Clontech), immediately upstream of the promoterless GUS gene. *Agrobacterium* LBA4404 cells harboring recombinant pBI101 plasmid were then used to transform tobacco cells with the standard leaf disk method [8]. For each *Ltp3* promoter–GUS construct, five tobacco transformants were selected for the histochemical and fluorogenic GUS assays [9]. In the histochemical assay, GUS expression was visualized in the leaf trichomes of transgenic tobacco plants containing *Ltp3*-1548, *Ltp*3-1143, and *Ltp3*-614 constructs (Fig. 3). GUS expression was also observed in the leaf peripheral epidermis and vascular veins of the transgenic plants containing *Ltp3*-1548–GUS. No GUS activity was detected in the leaves of tobacco transformants containing *Ltp3*-299 or *Ltp3*-148 (figure not shown but similar to the wild type) and the wild type tobacco plant (Fig. 3). These results indicate that the three

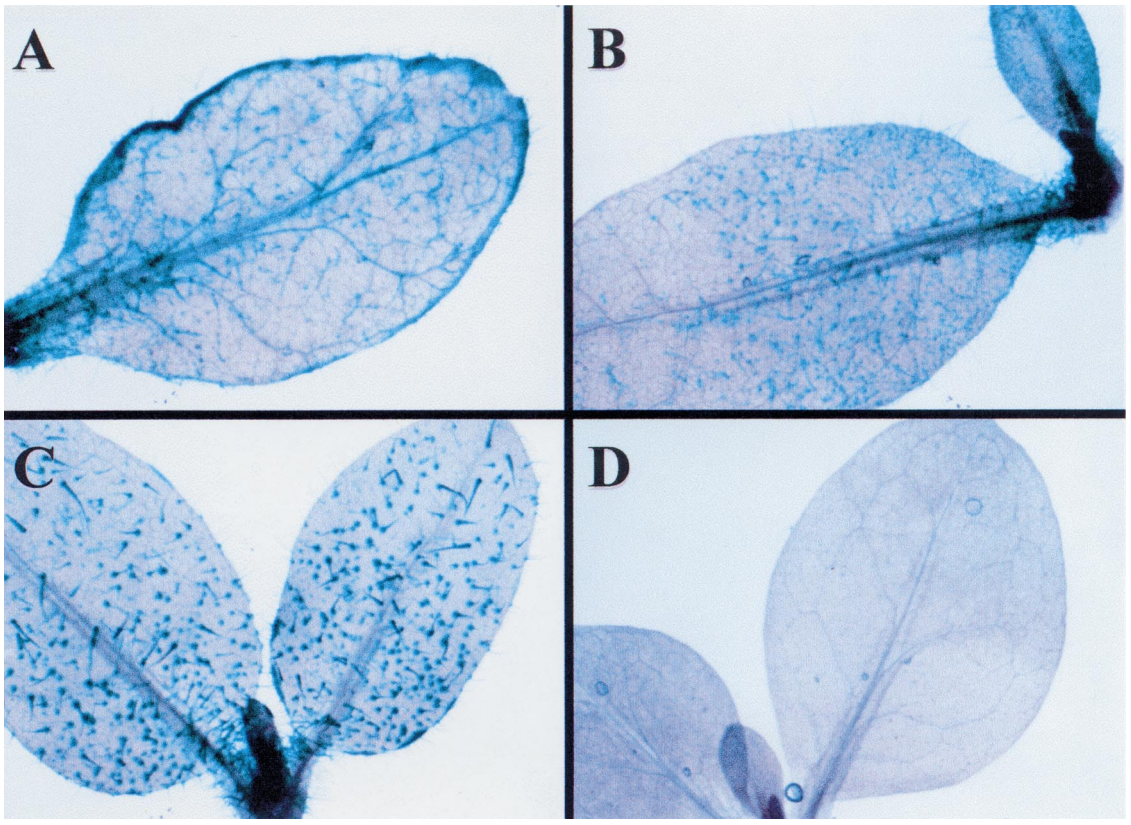

Fig. 3. Histochemical analysis of GUS gene expression in leaves of wild type and transgenic tobacco plants. *Ltp3*-1548 (A), *Ltp3*-1143 (B), and *Ltp3*-614 (C) direct GUS gene expression in the leaf trichomes of transgenic tobacco plants. No GUS expression was detected in the wild type tobacco plant (D).

Ltp3 promoters, *Ltp3*-1548, *Ltp3*-1143, and *Ltp3*-614, possess trichome-specific promoter activities, and that the basal promoter elements, TATA and CAAT boxes, in *Ltp3*-299 are not sufficient to direct GUS expression in transgenic tobacco plants. The results of fluorogenic GUS assay, based on the quantitation of 4-methylumbelliferone (4-MU) production (Table 1), indicate that *Ltp3*-1548, *Ltp3*-1143, and *Ltp3*-614 have strong promoter activities at similar levels in transgenic tobacco plants. However, *Ltp3*-299 and *Ltp3*-148 have very low promoter activities in tobacco transformants. The histochemical and fluorogenic results suggest that *cis*-elements conferring trichome specific activity of the *Ltp3* promoter are

Table 1
Fluorogenic GUS assays in tobacco plants

Gene construct	GUS activity[a]					S.E.M. (pmol 4-MU/h μg)
	A	B	C	D	E	
Ltp3-1548–GUS	19.2	19.1	27.0	18.3	50.5	28.8 ± 6.1
Ltp3-1143–GUS	37.1	30.1	23.8	12.1	20.8	24.8 ± 4.2
Ltp3-614–GUS	27.8	57.3	18.2	23.6	15.6	28.5 ± 7.5
Ltp3-299–GUS	0.08	0.07	0.10	0.09	0.08	0.08 ± 0.01
Ltp3-148–GUS	0.06	0.08	0.09	0.07	0.06	0.07 ± 0.01
CaMV 35S–GUS	190.2	378.0	215.7	236.8	361.2	276.4 ± 38.9
Wild type	0.07	0.05	0.08	0.06	0.07	0.07 ± 0.01

[a]GUS activities in five plants A, B, C, D, and E are expressed as pmol 4-MU/h μg.

located within a 315 bp DNA fragment, nt −614 to −300 relative to the translational start codon ATG of the *Ltp3* gene.

We had previously reported promoter activity of the cotton fiber specific gene *Ltp6* in transgenic tobacco [10]. The promoter activity of *Ltp6* appears weaker than that of *Ltp3* based on Northern analysis [6] and the histochemical and fluorogenic GUS assays [10]. Both *Ltp3* and *Ltp6* promoters can direct trichome-specific GUS expression in transgenic tobacco, suggesting that cotton fibers and tobacco trichomes share some ontogenetic similarities. The specificity and activity of *Ltp3* promoter need to be confirmed in transgenic cotton plants by further experiments. Some putative *cis*-elements have been found in the 5′ flanking region of the *Ltp3* gene. These include two G-box-like elements (CACGTAGA, nt −347 to −340; CACGTAGG, nt −242 to −235) and six CE1-like elements (CACC, nt −1490 to −1487, −128 to −125, and −68 to −65; GGTG, nt −1239 to −1236, −489 to −486, and −193 to −190), which have been shown to be involved in ABA responsiveness [11]; five E-box like elements (CAAATG, nt −376 to −371 and −285 to −280: CAATTG, nt −507 to −502; CAGTTG, nt −230 to −225; CATGTG, nt −819 to −814) recognized by the basic-region helix-loop-helix (bHLH) proteins [12] and involved in plant–pathogen interactions [13]; a CATGTAA (nt −711 to −705) sequence which is present in barley aleurone cell-specific genes [14]; and four putative recognition sites (CAGTTG, nt −230 to −225; GTTTGTT, nt −26 to −20; GGATA, nt −527 to −523; CTCCTACC, nt −98 to −91) for MYB-related transcriptional activators [15–19]. The 315 bp promoter DNA fragment (nt −614 to −300) has been radiolabeled by ^{32}P and used in an electrophoretic mobility shift assay (EMSA) [20] to bind cotton fiber nuclear proteins. Two forms of protein–DNA complexes were found to have slower mobility than the free DNA probe (data not shown), suggesting that transcription factors are capable of binding the *cis*-elements located in the 315 bp promoter region and control the transcription of the *Ltp3* gene.

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